EXAMINING THE EFFECT OF TKS5 SH3 DOMAIN MUTATIONS ON INVADOPODIA LOCALIZATION AND DEVELOPMENT IN CANCER CELLS

by

Kelley Elizabeth Whitaker

Honors Thesis

Appalachian State University

Submitted to The Honors College
in partial fulfillment of the requirements for the degree of

Bachelor of Science

August, 2015

Approved by:

Darren Seals, Ph.D., Thesis Director

Nicholas Kenny, Ph.D., Second Reader

Leslie Sargent Jones, Ph.D., Director, The Honors College

ABSTRACT

Examining the Effect of Tks5 Domain Mutations on Invadopodia Localization and Development in Cancer Cells

Kelley Whitaker

Honors Thesis Director: Dr. Darren Seals

Metastasis, the process by which cancers spread from their site of origin to distant anatomic sites, accounts for about ninety percent of all cancer deaths. Metastasis occurs when cancer cells acquire the invasive behaviors necessary for breaching tissue boundaries. Invadopodia, actin-rich cell surface protrusions, stimulate metastasis by enabling adhesion, motility, and extracellular matrix remodeling by cancer cells. Src tyrosine kinase and its substrate Tks5 localize to invadopodia and regulate their development. Tks5 is composed of a PX domain followed by five SH3 domains. While the lipid-binding PX domain is essential for Tks5 dependent invadopodia localization and development, the five protein-binding SH3 domains of Tks5 are less defined.

Since the general function of an SH3 domain is to provide a binding site for polyproline motifs in other proteins, we hypothesize that mutations within the SH3 domains of Tks5 would block the protein-protein interactions necessary for invadopodia localization and/or development in cancer cells. To study the role of these Tks5 SH3 domain mutations in invadopodia development, constructs containing inactivating point mutations (tryptophan to alanine) in each SH3 domain were shuttled into the expression vector pcDNA3.

Expression of wild-type and mutant Tks5 protein was confirmed in electroporated LNCaP prostate cancer cells. These Tks5 SH3 domain mutations showed variable effects on

invadopodia development in LNCaP cells, some of which accentuated and some of which inhibited the invadopodia-dependent matrix remodeling activity exerted by wild-type Tks5. These mutant Tks5 constructs are currently being used to determine localization in invadopodia-competent Src-transformed fibroblasts.

DEDICATION

This thesis is dedicated in loving memory of Adele Aliberti and the Right Reverend O'Kelley Whitaker.

ACKNOWLEDGEMENTS

This honors thesis would not have been possible without the continuous support of several people. First, I would like to thank Dr. Darren Seals for his guidance as a mentor throughout my time in his lab. The amount of knowledge that I have gained from this experience is invaluable. I would also like to thank Dr. Nicholas Kenny for his assistance as a second reader for my thesis. I have also appreciated the assistance of the other undergraduates and graduate students in this lab, specifically Christina Daly, Joey Breeyear, and Travis Tabor. In addition, I express my gratitude to the Honors College at Appalachian State University for supporting my research with an Honors College Partnership Board Research Fund Award. Lastly, I would like to thank my friends and family for their love and encouragement throughout the pursuit of this achievement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The War on Cancer

The National Cancer Act in 1971 marked a beginning in the war on cancer – a battle that seems to become more complicated with each diagnosis and scientific discovery (Burke 2004). The term "cancer" defines the many diseases that arise from mutations in cells that cause them to proliferate uncontrollably and to spread to other areas of the body. Approximately one in three women and one in two men will develop cancer in their lifetimes (Burke 2004). In 2014 alone, there were approximately 1,665,540 new cancer diagnoses and 585,720 cancer deaths, making it the second most common cause of death in the United States following heart disease (Siegel et al. 2014). The battle against cancer has proved to be very complicated. A main reason for this is that the pathway(s) to cancer development in each individual can vary greatly. The tremendous heterogeneity in cancer causality makes it extremely problematic to target and to treat (Hanahan and Weinberg 2000). There are, however, several hallmarks of cancer that are shared by most, if not all, cancers. These include: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming energy metabolism, and evading immune destruction (Hanahan and Weinberg 2011).

Much cancer research and clinical care has been directed at the surgical removal, irradiation, and/or chemotherapeutic treatment of cancerous cells. However, solid tumors, encompassing as much as one billion cells, are not the main cause of death among cancer patients (Burke 2004). Over 90% of all cancer-associated deaths are the consequence of a

process called metastasis. Metastasis is the result of cancer cells acquiring the invasive behavior necessary to breach tissue boundaries (Hannahan and Weinberg 2000). Once in the blood stream or lymph vessels, these metastasizing cancer cells can spread throughout the body and colonize vital organs such as the bones, liver, lungs, and brain (Burke 2004), allowing for more advanced stages of cancer to develop.

Relationship between Invadopodia, Src, Tks5, and Cancer

Podosomes and invadopodia are actin-rich cell surface protrusions that play a role in extracellular matrix remodeling and invasion (Murphy and Courtneidge, 2012) (Figure 1). The term *podosome* is used to characterize the structures of normal invasive cell types as regulated tissue invasion in the body is necessary for many biological processes including wound healing, organ development, immune system function, and inflammatory responses. The term *invadopodia* is used for the podosome-like invasive structures of cancer cells. Invadopodia enable invasion because they stimulate adhesion, motility, and extracellular matrix remodeling and degradation by cancer cells (Gimona et al. 2008). It has been speculated that invadopodia drive cancer cell invasion in the context of tumor metastasis.

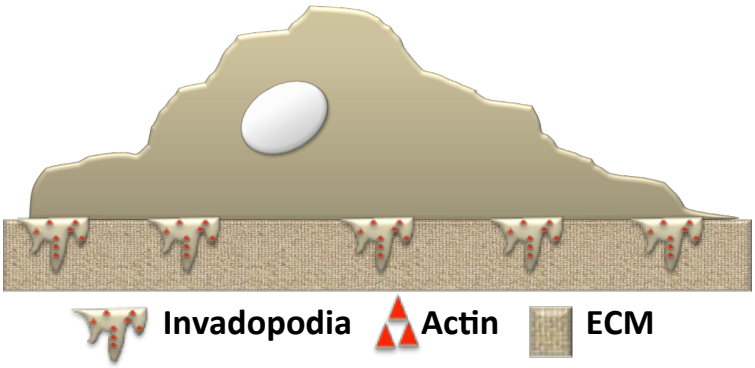

Figure 1. Depiction of Invadopodia. Invadopodia are actin-rich cell surface protrusions that extend into the underlying extracellular matrix (ECM) and support ECM remodeling by cancer cells.

Invadopodia have been found in human cancer cell lines as well as Src-transformed fibroblasts (Murphy and Courtneidge, 2012). Src, the first identified retroviral oncogene (Martin 2001), is a membrane-associated, non-receptor protein tyrosine kinase that has a role in some of the advanced stages of human cancers (Courtneidge et al. 2005). Among other factors, Src tyrosine kinase and its substrate Tks5 localize to invadopodia and regulate their development, though how they accomplish this has not been fully defined. Tks5 is an adaptor protein comprised of an amino terminal phox (PX) homology domain followed by five Src homology 3 (SH3) domains (Lock et al. 1998) (Figure 2). The PX domain of Tks5

Figure 2. Tks5 Modular Design. Tks5 is comprised of an amino-terminal PX domain followed by five SH3 domains. The organization of the domains within Tks5 is not shown to scale.

is known to bind to phosphatidylinosidol-3-phosphate PtdIns(3)P and PtdIns(3,4)P_2 (Abram et al. 2003; Oikawa et al. 2008). Moreover, invadopodia formation is dependent upon this phosphoinositide-binding property of the PX domain (Abram et al. 2003; Oikawa et al. 2008). In Src-transformed fibroblasts, the PX domain is targeted to invadopodia, and this domain is both necessary and sufficient for the invadopodia localization of Tks5 (Abram et al. 2003; Oikawa et al. 2008). Site-selective mutation or full deletion of the PX domain disables binding of Tks5 to membranes and presumably disables the formation of the Tks5-containing membrane protein complex necessary for invadopodia development (Abram et al. 2003; Courtneidge et al. 2005).

Tks5 directly regulates matrix-remodeling invadopodia activity. Silencing of Tks5 with shRNAs disables the ability of Src-transformed fibroblasts to form invadopodia, degrade a gelatin matrix, and invade through a matrix barrier comprised of Matrigel (Seals et al. 2005). Conversely, ectopic expression of wild-type Tks5 construct in the LNCaP prostate cancer cell line, a cell line that has low levels of Tks5 and does not form invadopodia, is sufficient to induce invadopodia-associated matrix remodeling and invasion activity (Burger et al. 2014). In this study we evaluated the use of model Src-transformed fibroblasts and LNCaP cells to determine the role of Tks5 SH3 domain mutations in the development of invadopodia.

Tks5 SH3 Domains and Invadopodia Development

Tks5 has five, tandem SH3 domains (Figure 2). In general, the primary function of SH3 domains is to mediate protein-protein interactions (Nguyen et al. 1998). These domains are comprised of two β sheets that contain three variable loops. One of the loops contains two, adjacent aromatic tryptophan residues that renders the SH3 protein eligible for binding to polyproline motifs. A point mutation at the first tryptophan residue is known to change the overall secondary structure of the SH3 domain and therefore inhibit protein-protein interactions (Tanaka et al. 1995). Currently, the polyproline-containing proteins that may bind to Tks5 SH3 domains are largely unknown though there is evidence that Tks5 interacts with ADAMs family metalloproteinases, N-WASp, WIP, and F-actin. Members of the ADAMs (a disintegrin and metalloproteinase) family (Weaver 2006) are known to interact with Tks5 (Abram et al. 2003). Specifically, ADAMs 12, 15, and 19 interact with Tks5 and ADAM19 binds to the fifth SH3 domain (Abram et al. 2003). There is also evidence supporting N-WASp (Neural Wiskott-Aldrich Syndrome Protein) binding to all five SH3

domains, WIP (WASP-interacting protein) binding to the third and fifth SH3 domains, and F-actin binding to the fifth SH3 domain of Tks5 (Weaver 2006, Oikawa et al. 2008, García et al. 2012). Disrupting the binding of N-WASp to the SH3 domains of Tks5 has been shown to decrease invadopodia formation (Oikawa et al. 2008). WIP is known to localize to invadopodia, and a recent study found that there is a correlation between decreased levels of WIP expression in cancer patients and improved prognosis (García et al. 2012). Using Tks5 mutants that contain a predicted inactivating point mutation in each of the five SH3 domains may allow for a better understanding of the role of these domains in the ability of Tks5 to control cancer cell invasion and metastasis.

Objectives

In this study, we proposed to further explore the function of the Tks5 SH3 domains in invadopodia development. Tks5 constructs were generated harboring point mutations in each of the five SH3 domains of Tks5. These constructs were then shuttled into the mammalian expression vector pcDNA3 to ensure robust, ectopic expression of Tks5. Both wild-type and mutant Tks5 constructs were then introduced into LNCaP cells to determine their role in inducing invadopodia activity. They were also introduced into Src-transformed fibroblasts to study their localization to invadopodia. This included a determination of their proper cell staining conditions for identifying the localization of ectopic Tks5. Together, these assays help to pinpoint which SH3 domains are important for invasive cancer phenotypes.

MATERIALS AND METHODS

Cell Culture

LNCaP cells (ATCC) were cultured at 37°C and 5% CO_2 in RPMI-1640 media (Thermo Scientific Hyclone, Logan, UT) formulated with 2.05 mM L-Glutamine, 110 mg/L sodium pyruvate, 10 mM HEPES and supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), and 1% penicillin/streptomycin. The cells were routinely subcultured every 3-4 days at 1:6 dilution.

Src-transformed fibroblasts (hereafter Src3T3 cells) were cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO) formulated with 4500 mg glucose/L, 110 mg sodium pyruvate/L, 4 mM L-glutamine and supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were routinely subcultured every 2-3 days at 1:20 dilution.

Cloning

Tks5 constructs harboring mutations in each of their respective SH3 domains had been previously generated in the mammalian expression vector pSGT. To generate constructs harboring these same mutations in the mammalian expression vector pcDNA3, we took advantage of the fact that a wild-type version of Tks5 already existed in this destination vector. Thus, small segments surrounding the mutant SH3 domains were cut from pSGT and used to replace the same segment in the wild-type Tks5 construct in pcDNA3 thereby converting wild-type Tks5 to each SH3 domain mutant (Figure 3A). Four mutant SH3 domain constructs for each of the first 4 SH3 domains of Tks5 (M1, M2, M3, and M4) were

generated in this fashion. In brief, mutant Tks5 constructs were cut with BlpI and BstEII (M1, M2, and M3) or BstEII and BsrGI (M4), and following purification on 1% agarose/0.5XTBE gels (Qiagen #28704), were ligated to the compatible sites generated by restriction of pcDNA3 with the same enzymes (Figure 3B and 3C). Ligation reactions used T4 DNA ligase (400 units, New England BioLab, #M0202S) at a 3:1 molar ratio (insert:vector) at 16˚C for overnight. Ligations were then transformed by heat-shock into NEB5α cells (New England BioLabs, #C2987H) according to manufacturer guidelines. Putative positive transformants grew as ampicillin resistant colonies on LB plates after overnight culture, were mini-prepped (Qiagen, #28704), and then sent to GeneWiz for DNA sequencing analysis to confirm the successful cloning of the mutated Tks5 construct. All DNA samples were quantitated on a Nanodrop (NanoDrop 1000 Spectrophotometer) according to manufacturer guidelines.

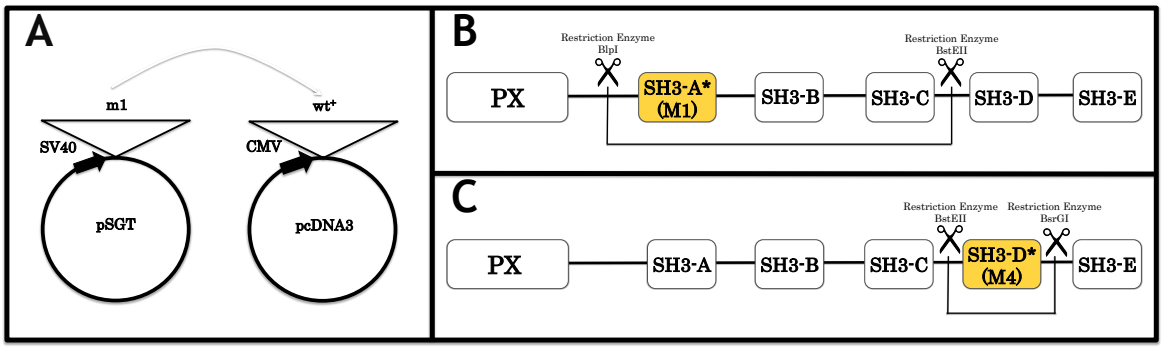

Figure 3. Cloning Strategies for Tks5 SH3 Domain Mutants. (A) Diagram depicting the general strategy for moving Tks5 mutations from pSGT to pcDNA3. SV40 and CMV are promoters. (B and C) Cloning strategies involving the indicated restriction enzymes (BlpI and BstEII for M1, M2, and M3; BstEII and BsrGI for M4) were used to replace regions of wild-type Tks5 in pcDNA3 with similar regions from the mutant SH3 domain constructs of pSGT.

Sequencing

Tks5 SH3 domain mutant constructs subcloned into the expression vector pcDNA3 were verified by DNA sequencing using regional primers. DNA sequencing using the vector-specific primer SP6 were used to verify the inclusion of a C-terminal myc tag in certain pcDNA3-Tks5 constructs. All sequencing reactions were carried out by GeneWiz where vector constructs (800 ng) were mixed with primers (25 pmol) in a final volume of 15μL according to company guidelines. Primers used in this study are listed in Table I.

Table I. Primers used in this study.

Primer	Application	Sequence
Asuprimer4	Forward primer for M1 and M2 sequencing	5'- GAAGTCTTCCGGTTCTTTGAG -3'
Asuprimer5	Forward primer for M3 sequencing	5'- GAGAGGACCACATCCAAGCTA -3'
Asuprimer6	Forward primer for M4 sequencing	5'- AGGGCTCTAGGAGTGAGGACT -3'
SP6	Reverse primer for Myc tag sequencing	5'- GATTAAGGTGACACTATAG -3'

Electroporation

LNCaP and Src3T3 cells (2 x 10^6) were mixed with pSGT or pcDNA3 mammalian expression vector constructs (3-8μg) and Amaxa™ Cell Line Nucleofector™ Kit R (Lonza, Basel, Switzerland) solution in a final volume of 100μL. Electroporations were carried out using the Amaxa™ Nucleofector™ 2b (Lonza, Basel, Switzerland) programs T-009 (LNCaPs cells) and X-001 (Src3T3 cells). Following the electroporation, the cells recovered in pre-warmed media for 15-30 minutes. Cells were then plated in 6- or 12-well plates containing uncoated glass coverslips and incubated at 37°C for 24-48 hours. Src3T3 cells were incubated on glass coverslips for fluorescent microscopy studies.

Immunoblotting

Cell lysates were prepared from cells grown in 6-well culture plates under described culture conditions. All plates were kept on ice and washed twice in 1.2μL of ice-cold phosphate-buffered saline (PBS) and 1mM sodium orthovanadate. Cells were then lysed with 100μL of ice-cold 1% NP-40 lysis buffer composed of 20mM Hepes (pH=7), 110mM sodium chloride, 40mM sodium fluoride, 1% NP-40, 1mM sodium orthovanadate, and the following protease inhibitors: 10μg/mL aprotinin, 10μg/mL benzamidine, 10μg/mL leupeptin, 10μg/mL pepstatin, and 1mM PMSF. The cells were then scraped from each dish with a Corning 3008 Cell Lifter, transferred to ice-cold microfuge tubes, vortexed for 5 seconds, and incubated on ice for 10 minutes. Lysates were then centrifuged at 10,000x*g* for 10 minutes at 4°C to pellet cellular debris. Supernatants were then transferred to new ice-cold microfuge tubes and assayed directly for protein concentration using a detergent-compatible protein assay kit (Bio-Rad) with bovine serum albumin (BSA) as a standard. Lysates were diluted in SDS loading buffer at a concentration of 0.5μg/μL or 1μg/μL and heated at 95°C for 5 minutes before being loaded on a 7.5% polyacrylamide gel and undergoing protein separation by SDS-PAGE. The polyacrylamide gel was run on a PowerPac™ HC High-Current Power Supply (Bio-Rad) at 100V for 15 minutes and then 150V for 60 minutes. Proteins were then transferred from the polyacrylamide gel to nitrocellulose membranes using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) for 35 minutes at 0.22 Amps. All immunoblots were blocked in 5% dry milk and 0.5% BSA in PBS containing 0.1% Tween-20 (PBST) and 0.05% sodium azide (pH = 7) for 60 minutes. Primary antibodies were then applied to the membranes and incubated at 4°C overnight. Immunoblotting primary antibodies included: Myc (1:1000; 9E10, VARI or 4A6, #05-724,

Millipore), GAPDH (1:1000; #sc-25778; Santa Cruz), and Tks5 (1:1000; #sc-31022; Santa Cruz) in 10% blocking solution containing 0.05% sodium azide. After three 10 minute washes in PBST, the appropriate species-specific secondary antibody conjugated to horseradish peroxidase (1:1000 and 1:2000; NA931V and NA934; GE Healthcare) was applied to the membranes for a minimum of 30 minutes at room temperature. Immunoblots were developed using a Western Lightning Plus Chemiluminescence reagent (Perkin Elmer) according to manufacturer's guidelines and then imaged using a ChemiDoc imaging system (Bio-Rad).

Microscopy

Cells were grown on glass cover slips in 12-well plates under described culture conditions. Coverslips were fixed in 1ml 3% formaldehyde (Electron Microscopy Sciences) in PBS for 10 minutes, washed three times in 1mL PBS, permeabilized in 1mL 0.4% Triton X-100 for 4 minutes, and then washed three times in 1mL PBS again. Primary antibodies were used to detect the Myc epitope (1:333 and 1:1000; clone 9E10, VARI; clone 4A6, Millipore) in 5% donkey serum/PBS overnight at 4°C. Coverslips were then washed three times in 1mL PBS before being incubated with the appropriate fluorescent-conjugated secondary antibody (1:1000 and 1:2000; AlexaFlour 594-anti-mouse; A21203; GE Healthcare) and AlexaFluor 488-conjugated phalloidin (1:200, AlexaFluor 488 Phalloidin; A12379; Life Technologies) in 5% donkey serum/PBS at room temperature for 45 minutes. After another three washes in 1mL PBS, the coverslips were mounted onto glass slides with ProLong Gold plus DAPI (P36941; Life Technologies). Coverslips were incubated overnight at room temperature before sealing with nail polish. Images were captured with an Olympus

BX51 microscope equipped with a Retiga EXi Fast 1394 camera. Image processing was conducted with Q-Capture Suite software version 2.9.13. Images were captured at 100X magnification at exposure times of 197ms, 902ms, and 100ms for actin, myc, and nuclei respectively.

RESULTS

Previous research conducted in the laboratory of Dr. Seals has used Src-transformed fibroblast (Src3T3) and LNCaP prostate cancer cell lines to study the development and function of invadopodia. Src3T3 cells make invadopodia via the ectopic expression of constitutively active chicken Src(Y527F) (Furmaniak-Kazmierczak et al. 2007). They have high levels of endogenous Tks5 protein. LNCaP cells have low levels of Tks5 and do not form invadopodia (Burger et al. 2014). However, ectopic expression of a wild-type Tks5 construct is sufficient to induce invadopodia formation and invadopodia-associated matrix remodeling activity (Burger et al. 2014). Both cell lines are easily manipulated model systems for studying the processes necessary for invadopodia development.

Subcloning of Tks5 SH3 Domain Mutants into the pcDNA3 Vector

Prior to studying the function of the five Tks5 SH3 domain mutant constructs, we first wanted to demonstrate sufficient expression in these model cancer cell lines. First, the expression of wild-type murine Tks5 from either the pSGT vector (SV40 promoter) or pcDNA3 vector (CMV promoter) were compared following electroporation into LNCaP cells. After 48 hours, it was found that pcDNA-Tks5 exhibited much greater expression than pSGT-Tks5 (Figure 4). This suggested that the pcDNA3 vector might be a better choice in studies of invadopodia development in this cell line.

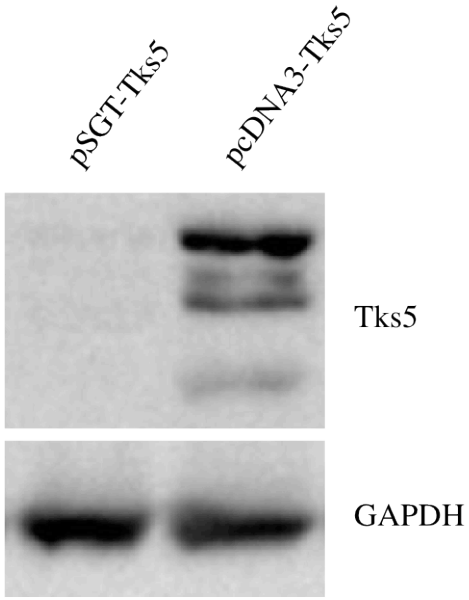

Figure 4. Effect of Promoter on Ectopic Tks5 Expression in LNCaP Cells. Immunoblot analysis of the expression of wild-type Tks5 constructs from the mammalian expression vectors pSGT and pcDNA3 following electroporation into LNCaP cells. GAPDH served as a loading control.

Previous research in the Seals lab had generated tandem nucleotide substitutions in the coding regions for each of the SH3 domains of murine Tks5. However, these constructs were present in the pSGT vector. Based on the results of Figure 4, we therefore decided to shuttle these SH3 domain mutants into pcDNA3 to allow for more robust expression of Tks5 in LNCaP prostate cancer cells (Figure 3). Different restriction enzymes were used to replace the wild-type Tks5 already present in the pcDNA3 vector with the mutated Tks5 constructs found originally in the pSGT vector. For mutant SH3 domains 1-3 (M1-M3), restriction enzymes BlpI and BstEII were used, and for mutant SH3 domain 4 (M4), restriction enzymes BstEII and BsrGI were used (Figures 3). Tks5 mutant SH3 domain 5 (M5) had already been generated in the pcDNA3 vector by prior researchers in the Seals lab.

Successful cloning of the first four Tks5 SH3 domain mutants was confirmed after performing a sequence analysis using the regional Tks5 primers (Table I). The nucleotide sequence of the wild-type Tks5 construct in pcDNA3 is designated by the sequence TGGTGG and encodes a pair of tryptophans (Figure 5). Each SH3 domain construct (M1-M4), however, demonstrated the successful acquisition of the tandem nucleotide substitutions (**GCG**TGG) that changed the first tryptophan to an alanine.

```
                                                  W   W
Nucleotide Sequence wt: … GAGAAGAACGAAAGCGGC TGGTGG TTTGTGAGCACATCTGAA …
Nucleotide Sequence M1: … GAGAAGAACGAAAGCGGC GCGTGG TTTGTGAGCACATCTGAA …

Nucleotide Sequence wt: … CGAAAGAACCTGGAAGGC TGGTGG TACATCAGGTACCTCGGC …
Nucleotide Sequence M2: … CGAAAGAACCTGGAAGGC GCGTGG TACATCAGGTACCTCGGC …

Nucleotide Sequence wt: … GACAAGAACTCGGGTGGT TGGTGG TACGTGCAGATCGGGGAG …
Nucleotide Sequence M3: … GACAAGAACTCGGGTGGT GCGTGG TACGTGCAGATCGGGGAG …

Nucleotide Sequence wt: … AGAAGGCGGTAAGTGGGG TGGTGG TACGTGAGGTTTGGGGAG …
Nucleotide Sequence M4: … GAGAAGGCGGTAAGTGGG GCGTGG TACGTGAGGTTTGGGGAG …
                                                  A   W
```

Figure 5. Annotated Sequence Analysis of Tks5 SH3 Domain Mutants. Shown are the DNA sequencing results for the four mutant constructs confirming the acquisition of the tryptophan (W) to alanine (A) substitutions. Sequencing was performed by GeneWiz using regional Tks5 primers.

Effect of SH3 Domain Mutants on Invadopodia Activity in LNCaP Cells

The Tks5 SH3 domain mutants are currently being electroporated into LNCaP prostate cancer cells to explore any alterations in their invadopodia-associated matrix remodeling properties when compared to wild-type Tks5. It was initially hypothesized that the single point mutation in each of the five SH3 domains would inactivate the domain that the mutation inhabits and that this would interfere with invadopodia development. However, Tks5 SH3 domain mutants M1-M3 have all been found to degrade gelatin more than the wild-type Tks5, where as M4 and M5 have been found to degrade gelatin less (Daly and

Seals, unpublished data). The reason for these differences is the emphasis of another project in the Seals lab. However, an immunoblot analysis has been used to confirm the expression of wild-type Tks5 and the five SH3 domain mutants (M1-M5) in these cells (Figure 6).

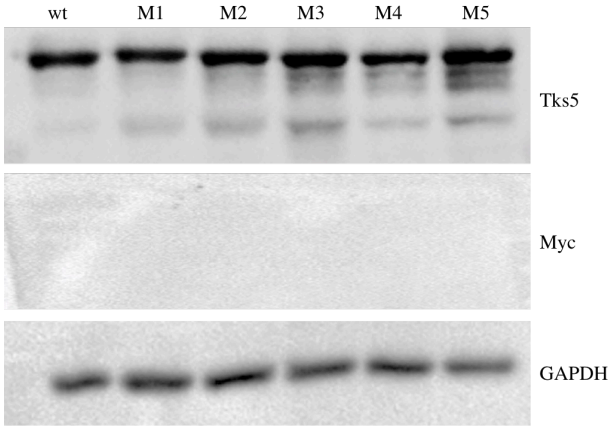

Figure 6. Immunoblot Analysis of Tks5 Mutant Constructs. Tks5 constructs (wild-type, wt; SH3 domain mutants, M1-M5) were electroporated into LNCaP cells, and then analyzed 48 hours later for Tks5 protein levels using either a Tks5 antibody or an antibody to the Myc epitope. GAPDH served as a loading control

Effect of SH3 Domain Mutants on the Localization of Tks5 in Src3T3 Cells

One hypothesis regarding the differential effects of Tks5 on invadopodia development in LNCaP cells is that the mutations in the SH3 domains will impact invadopodia localization. To address this, we have used fluorescent microscopy to study Tks5 localization in model Src3T3 cells. One caveat to these studies is that model invadopodia-competent cell lines like Src3T3 cells have abundant endogenous Tks5 expression. To study the localization of the mutant Tks5 constructs, we needed to use constructs that harbor epitope tags. Past work in the Seals lab had generated such constructs with the Myc epitope added to the 3' end of the coding sequence. Thus, when Myc was stained with a specific fluorescent antibody, we could be sure we were localizing the mutant Tks5 constructs, not the endogenous protein. We first wanted to confirm the presence of the Myc tag in the wild-type and mutant Tks5 constructs from pcDNA3 used previously in the

invadopodia activity studies in LNCaP cells by performing immunoblot analysis and DNA sequencing. By immunoblot analysis, Tks5 was clearly being expressed, but there was no ability to detect Tks5 with an antibody to the Myc epitope (Figure 6). Moreover, using the SP6 primer derived from vector sequences, DNA sequencing confirmed that the Tks5 constructs in pcDNA3 did not have the Myc epitope (Figure 7).

```
Tks5-myc in pcDNA3 (52): … AACTACCTTGAGAAGAAGAACGGGGAGCAGAAGCTGATNTCGGAGGAGACNNNN
Tks5 in pcDNA3 (2):      … AACTACCTTGAGAAGAAGAACTAATAGCACAGGGTCCTTCCAAGACTC …
Tks5-M5 in pcDNA3 (20):  … AACTACCTTGAGAAGAAGAACTAATAGCACAGGGTCCTTCCAAGACTC …
Tks5-M1 in pcDNA3 (378): … AACTACCTTGAGAAGAAGAACTAATAGCACAGGGTCCTTCCAAGACTC …
```

Figure 7. Determining the Presence of the Myc tag on Tks5 Constructs. All indicated Tks5 constructs were sequenced at the 3' end of the insert using the vector-specific primer SP6. Only the Tks5-myc construct in pcDNA3 contained the Myc epitope (green). TAA (red) is a stop codon. Vector sequences from pcDNA3 are blue. Nucleotides indicated by "N" are unidentified. The numbers following each construct, in parenthesis, represent their stock numbers within the Seals lab archives.

We further examined for the presence of the Myc epitope by analyzing Src3T3 cells electroporated with wild-type Tks5 constructs from pcDNA3 and pSGT that were known to contain this tag (Figure 8). Both vectors demonstrated Tks5 expression at very similar levels using an antibody to the myc epitope. The Tks5-myc in the pcDNA3 vector also showed a light band slightly above the darker band that is in both pcDNA3 and pSGT lanes. It is unknown what the lighter band represents.

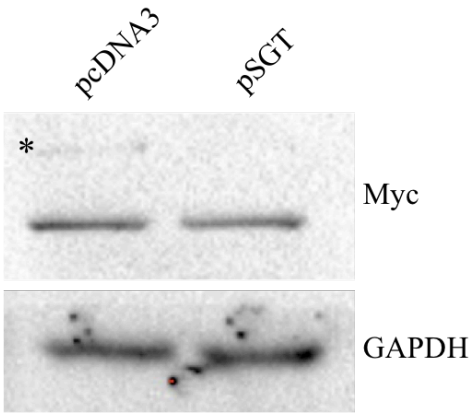

Figure 8. Immunoblot Analysis of Tks5-myc Constructs. Tks5-myc constructs from pcDNA3 and pSGT vectors were electroporated into Src3T3 cells and protein levels were analyzed by immunoblot analysis using an antibody to the Myc tag. Comparable protein levels are shown for Tks5-myc in both of these vectors, though a lighter band at higher molecular weight (*) can also be seen in the pcDNA3 construct. GAPDH served as a loading control.

In order to prepare for the localization experiments using the Tks5 SH3 domain mutant constructs, we first determined proper staining conditions using Src3T3 cells with or without electroporation of wild-type Tks5-myc. Both cells were stained with and without phalloidin to visualize F-actin, with and without a primary myc antibody (1:333), with a mouse-specific secondary antibody (1:1000), and with DAPI to visualize nuclei. The secondary antibody controls with and without phalloidin (Figure 9, panels A-F and panels M-R) verified that the signal we see in the red channel of the microscope when staining with the primary Myc antibody (Figure 9, panels G-L and panels S-X) was not due to non-specific binding by the secondary antibody or from bleed-through of F-actin staining in the green channel. Use of the Myc antibody increased the signal in the red channel of the Src3T3 cells electroporated with Tks5-myc as expected (Figure 9, panels H and K). Signal was present throughout the cytoplasm of the cells, but also appeared to be strong in actin-rich invadopodia (arrows). However, we also detected significant signal with the Myc antibody in Src3T3 cells that were not electroporated with Tks5-myc (Figure 9, panels T and W).

However, it does appear that the localization was confined to the cytoplasm with few invadopodia detected.

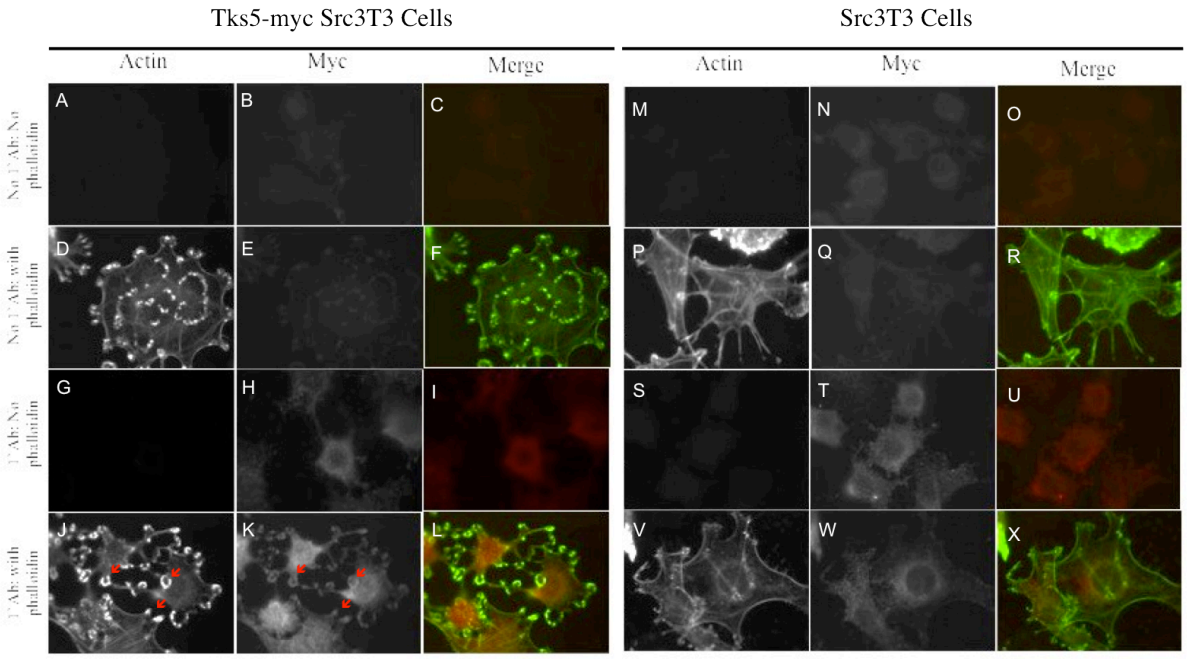

Figure 9. Localization of Tks5-myc in Src3T3 Cells. Tks5-myc Src3T3 cells and Src3T3 cells were stained under four different conditions: with and without AlexaFluor 488-phalloidin (1:200) and with and without a primary Myc antibody (4A6; 1:333). All conditions were stained with a secondary antibody (AlexaFlour 594-anti-mouse; 1:1000). Images were taken at 100X magnification.

DISCUSSION

In this study, attempts were made to ascertain the role of SH3 domain mutants in the localization of Tks5 to invadopodia and the impact of Tks5 on invadopodia activity. To identify a role for Tks5 in invadopodia activity, mutant SH3 domain constructs in the pSGT vector were shuttled over to the pcDNA3 vector because the latter provided much better expression in LNCaP cells. The successful cloning of the SH3 domain mutants M1-M4 was verified by DNA sequencing, while the expression of these mutants in the LNCaP prostate cancer cell line was verified by immunoblot analysis. At this point, preliminary data suggests differential effects by the SH3 domain mutants on the ability of LNCaP cells to degrade gelatin monolayers, a measure of invadopodia-associated invasion. It was initially hypothesized that an inactivating point mutation in one of the SH3 domains would lessen the invasive behavior of the LNCaP prostate cancer cells relative to wild-type Tks5. It was shown, however, that Tks5 mutants M1-M3 accentuated gelatin degradation whereas mutants M4 and M5 inhibited gelatin degradation (Daly and Seals, unpublished data). The reason for the variable change in the invasive qualities of the LNCaP cells is unknown. One thought is that the change in protein interactions in the mutated Tks5 constructs can alter the ability of Tks5 to be phosphorylated by Src, and hence Tks5 activity. This could lead to smaller or larger concentrations of Tks5 being phosphorylated in cells and therefore a change in the amount of active Tks5 in the cells. Further studies will address this hypothesis.

The primary focus of this study was to address the localization of Tks5 in invadopodia-competent Src3T3 cells. Unfortunately, Src3T3 cells, like many other model cell lines that are able to form invadopodia, have an abundance of endogenous Tks5. We

initially thought that we could use the same constructs used in the invadopodia activity assays described above because they harbored a Myc epitope tag on the 3' end of the coding sequence. However, both immunoblot analysis and DNA sequencing confirmed the absence of this epitope in the pcDNA3 constructs. It is thought that the pSGT constructs do contain the Myc epitope tag, thus we have thus turned to the pSGT constructs for these localization studies.

To study the localization of mutated Tks5 constructs in Src3T3 cells, proper staining conditions must be established using wild-type, Myc-tagged Tks5. To this end, Src3T3 cells electroporated with pSGT Tks5-myc were cultured on glass coverslips and then stained with fluorescently-conjugated phalloidin, a myc antibody, a fluorescent conjugated secondary antibody, and DAPI to stain for actin, myc, and nuclei respectively. Src3T3 cells without Tks5-myc were also cultured on glass coverslips and stained using the same conditions as a control. The staining of the Tks5-myc Src3T3 cells gave good signals. Phalloidin stained the actin filaments and revealed numerous punctate and rosette-shaped invadopodia. Myc could also be seen in the cells, mostly in the cytoplasm, but there was also signal in the invadopodia as well. It is known that Tks5 localizes to invadopodia, so seeing the Myc signal there was expected.

Control Src3T3 cells were similarly stained with phalloidin and the Myc antibody and gave similar signals as the Src3T3 cells electroporated with Tks5-myc. This was unexpected, and suggests that the Myc antibody may need further titration. However, we did note that there was less invadopodia staining by the Myc antibody in the control cells versus the cells that were elctroporated with Tks5-myc. Further efforts are needed to verify expression of

these constructs in these cells and in the development of the best staining conditions to reveal localization of ectopic Tks5.

FUTURE DIRECTIONS

Determining where each Tks5 SH3 domain mutant localizes to in invadopodia-competent Src3T3 cells will help clarify the role of Tks5 in invadopodia development as well as the ability of cancer cells to invade and metastasize. This work, alongside gelatin degradation assays and assays of invasion and Tks5 phosphorylation will determine how active the mutated Tks5 is in these cancer cells, which would bring to light the role of Tks5 in tumors as well.

REFERENCES

Abram CL, Seals DF, Pass I, Salinsky D, Maurer L, Roth TM, Courtneidge SA. 2003. The Adaptor Protein Fish Associates with members of the ADAMs Family and Localizes to Podosomes of Src-transformed Cells. The Journal of Biological Chemistry **278**: 16844-16851.

Burger KL, Learman BS, Boucherle AK, Sirintrapun SJ, Isom S, Días B, Courtneidge SA, Seals DF. 2014. Src-dependent Tks5 phosphorylation regulates invadopodia-associated invasion in prostate cancer cells. The Prostate **74**: 134-148.

Burke D. 2004. Why We're Losing The War On Cancer [And How To Win It] [Avastin, Erbitux, Gleevec … The new wonder drugs might make you think we're finally beating this dreaded scourge. We're not. Here's how to turn this fight around.] Fortune.

Courtneidge SA, Azucena Jr. EF, Pass I, Seals DF, Tesfay L 2005. The Src Substrate Tks5, Podosomes (Invadopodia), and Cancer Cell Invasion. Cold Spring Harbor Symposia on Quantitative Biology **LXX**.

García E, Jones GE, Machesky LM, Antón IM. 2012. WIP: WASP-interacting proteins at invadopodia and podosomes. European Journal of Cell Biology **91**: 869-877.

Gimona M, Buccione R, Courtneidge SA, Linder S. 2008. Assembly and biological role of podosomes and invadopodia. Current Opinion in Cell Biology **20**: 235-241.

Hanahan D, Weinberg RA. 2000. The Hallmarks of Cancer. Cell **100**: 57-70.

Hanahan D, Weinberg RA. 2011. Hallmarks of Cancer: The Next Generation. Cell **144**: 646-674.

Furmaniak-Kazmierczak E, Crawley SW, Carter RL, Maurice DH, Côté GP. 2007. Formation of Extracellular Matrix-Digesting Invadopodia by Primary Aortic Smooth Muscle Cells. Circulation Research **100**: 1328-1336.

Lock P, Abram CL, Gibson T, Courneidge SA. 1998. A new method for isolating tyrosine kinase substrates used to identify Fish, an SH3 and PX domain-containing protein, and Src substrate. The EMBO Journal **17**: 4346-4357.

Murphy DA, Courtneidge SA. 2012. The 'ins' and 'outs' of podosomes and invadopodia: characteristics, formation and function. Nat Rev Mol Cell Biol **12**: 413-426.

Nguyen JT, Turck CW, Cohen FE, Zuckermann RN, Lim WA. 1998. Exploiding the Basis of Proline Recognition by SH3 and WW Domains: Design of N-Substituted Inhibitors. Science **282**: 2088-2092.

Oikawa T, Itoh T, Takenawa T. 2008. Sequential signals toward podosome formation in NIH-src cells. J. Cell Biol. **182**: 157-169.

Seals DF, Azucena Jr. EF, Pass I, Tesfay L, Gordon R, Woodrow M, Resau JF, Courtneidge SA. 2005. The adaptor protein Tks5/Fish is required for podosome formation and function, and for the protease-driven invasion of cancer cells. Cancer Cell **7**: 155-165.

Siegel R, Ma J, Zou Z, Jemal A. 2014. Cancer Statistics, 2014. A Cancer Journal for Clinicians **64**: 9-29.

Tanaka M, Gupta R, Mayer BJ. 1995. Differential Inhibition of Signaling Pathways by Dominant-Negative SH2/SH3 Adaptor Proteins. Molecular and Cellular Biology **15**: 6829-6837.

Weaver AM. 2006. Invadopodia: specialized cell structures for cancer invasion. Clinical and Experimental Metastasis **23**: 97-105.

BIOGRAPHICAL SKETCH

Kelley Elizabeth Whitaker was born at Bell View Women's Hospital in Niskayuna, New York to Barbara and John Whitaker. The family moved to Cary, North Carolina, where Kelley received her secondary education, graduating from Panther Creek High School in 2011. In August of 2011, Kelley moved to Boone to study Cell/Molecular Biology at Appalachian State University as a University Honors student. In fall of 2013, Kelley began studying cancer biology under the mentorship of Dr. Darren Seals in the Department of Biology at Appalachian State. In 2015, Kelley was awarded with the Honors College Partnership Board Research Fund Award, was chosen to participate in the Celebration of Student Research at Appalachian State University, and was acknowledged as a Meritorious Senior in the Biology Department. After completing her Bachelor of Science degree in Cell/Molecular Biology from Appalachian State University in August 2015, Kelley will be moving to Raleigh, North Carolina. Here she will begin her career as a Project Specialist in the Oncology division at INC Research, a clinical research organization.